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Virucidal activities of medium- and long-chain fatty alcohols and lipids against respiratory syncytial virus and parainfluenza virus type 2: comparison at different pH levels

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Summary

Recent studies have shown that some lipids and fatty alcohols have microbicidal activities against a broad variety of pathogens. In this study, virucidal activities of fatty acids, monoglycerides and fatty alcohols were tested against respiratory syncytial virus (RSV) and human parainfluenza virus type 2 (HPIV2) at different concentrations, times and pH levels. The most active compounds were mixed with milk products and fruit juices and the mixtures tested for virucidal effects. The aim was to determine which compounds are the most active against these respiratory viruses and could possibly be used in pharmaceutical formulations or as additives to milk products or juice. Several compounds caused a significant inactivation of virus, and there was generally a good agreement between the activities against RSV and parainfluenza virus. By changing the pH from 7 to 4.2, the virucidal activities of some of the compounds were greatly increased, i.e., they inactivated virus in a shorter time and at lower concentrations. The most active compound tested was 1-monoglyceride of capric acid, monocaprin, which also showed activity against influenza A virus and significant virucidal activities after addition to milk products and fruit juices, even at a concentration as low as 0.06–0.12%. The significant virucidal activities of fatty alcohols and lipids on RSV and parainfluenza virus demonstrated in this *in vitro* study raise the question of the feasibility of using such compounds as ingredients in pharmaceutical dosage forms against respiratory infections caused by these viruses, and possibly other paramyxo- and myxoviruses.

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Introduction

Human respiratory syncytial virus (RSV) and parainfluenza viruses are major causes of upper and lower respiratory tract infections. RSV especially is a leading cause of serious lower respiratory tract infections in infants and the elderly and also in immunocompromised individuals and those with pulmonary and cardiac disease [25]. It is estimated that 95% of children at the age of 2 years have been exposed to RSV [14], and there is evidence indicating that severe RSV infections in infants under 3

months of age are associated with recurrent wheezing, asthma and other pulmonary disorders in later childhood due to abnormal development of the immune system [7, 24, 25].

Ribavirin is the only chemotherapeutic agent available to treat RSV infections, with restricted use only in high-risk or severely ill infants, due to efficacy and safety concerns. No vaccines are currently licensed for prevention of RSV infections. Although the humanized monoclonal antibody, Palivizumab, and immunoglobulin products containing high neutralizing antibody titers to RSV, e.g., RSV-IVIG, have been found to be effective for protection against severe RSV infections, they are only recommended for prophylactic use in high-risk children [25], and questions have been raised about their relative cost-benefit ratio [35]. There is therefore a need for new prophylactic or therapeutic compounds for general use against RSV and other viruses causing respiratory infections.

Certain natural lipids have been shown to have a high antiviral activity and are being developed as antimicrobial ingredients in drug formulas that rapidly kill viruses and bacteria on contact [2, 17, 19, 29, 31, 32]. Studies showed that saturated medium-chain fatty acids and their corresponding monoglycerides and fatty alcohols rapidly inactivate viruses such as herpes simplex virus (HSV) and human immunodeficiency virus [12, 13, 15, 26, 27, 30, 33, 34]. Some of these lipids also have broad antibacterial activities against both Gram-negative and Gram-positive bacteria [3–6, 30].

In this study, we report *in vitro* virucidal activities of lipids against RSV and HPIV2, their increased effectiveness at low pH, and virucidal activities of milk, milk formulas and fruit juice mixed with such compounds.Their possible use to prevent or to ameliorate respiratory infections is discussed.

Materials and methods

Alcohols, fatty acids and monoglycerides

N-Decyl and lauryl alcohols were purchased from ICN Biomedicals Inc., Aurora, Ohio. All other alcohols and lipid compounds came from Sigma Chemical Co., St. Louis, MO (purest grade). Stock solutions of 1 M alcohols and fatty acids were made in ethanol (Merck).

Cell culture and media

Vero (African green monkey kidney cell line) and MA 104 (rhesus monkey kidney cell line) cells were grown in RPMI-1640 medium (Gibco) with 2.0 mM L-glutamine, 0.05 mg/ml gentamicin, 0.375% (w/v) sodium bicarbonate and 10% heat-inactivated fetal bovine serum (FBS). The maintenance medium (MM) was RPMI-1640 with 2% FBS. MDCK (Madin-Darby canine kidney cell line) cells were grown in MEM (Gibco) with 2.0 mM L-glutamine, 0.05 mg/ml gentamicin, 0.375% (w/v) sodium bicarbonate and 10% heat-inactivated FBS. The MM for MDCK cells was MEM with 2% FBS and HEPES (10 mM).

Virus and virus titration

RSV ATCC (strain Long, American Type Culture Collection, Rockville, MD) species: *Human respiratory syncytial virus*, genus: *Pneumovirus* and a member of the family *Paramyxoviridae*, was grown in Vero cells. Three recent clinical isolates of RSV, isolated from nasopharyngeal aspirates from infants and young children with respiratory infection, were grown in MA 104 cells and passed 5–6 times to increase the titer. HPIV2, species: *Human parainfluenza virus 2*, genus: *Rubulavirus* and another member of the family *Paramyxoviridae*, was isolated from bronchoalveolar lavage from a child with respiratory infection and passed 4 times in Vero cells before use in experiments. Influenza virus, species: *Influenza A virus*, strain: A/Iceland/32/2003 (H_1N_1), a member of the family *Orthomyxoviridae*, isolated from a child with respiratory infection, was passed 3 times in MDCK cells before use. The clinical isolates were obtained from the Department of Medical Virology, National University Hospital, Reykjavik, Iceland. Stocks of RSV, HPIV2 and influenza virus with infectivity titers of 10^4–10^5 $CCID_{50}$ (50% cell culture infective dose) per 100 µl were used in the experiments. Virus was titrated by inoculation of 10-fold dilutions in MM onto monolayers of cells in 96-well microtiter tissue culture plates (Nunc, Roskilde, Denmark). One hundred µl of each virus dilution was inoculated into quadruplicate wells. The plates with Vero and MA cells were incubated at 37 °C in a humidified incubator with 5% CO_2 in air and examined for cytopathic effect (CPE) for 10 days. MDCK cell plates were incubated for 1 h at 33 °C with influenza virus mixtures, and then the samples were replaced by MM containing 1.4 µg of Trypsin–TPCK (Fluka) per ml. The MDCK plates were incubated at 33°C in a humidified incubator with 5% CO_2 in air and examined for CPE for 10 days. Virus titers ($\log_{10}$ $CCID_{50}$/100 µl) were calculated by the method of Reed and Muench [20].

Assay of virucidal activity

Stock solutions of alcohols and lipids were diluted to the desired concentrations in MM or in milk products and fruit juices by vortexing at the highest speed for 30 sec at 37°C.

Each dilution was thoroughly mixed with an equal volume (100 µl) of virus in 65 × 15-mm round-bottom polystyrene tubes (Falcon). The mixtures were incubated at 37 °C in a water bath for the desired length of time, except for the 1-min incubation and influenza virus samples, which were incubated at room temperature. The action of the alcohols or lipids was stopped by diluting the mixtures 10-fold in MM. Virus mixed with MM and with MM containing ethanol at a final concentration of 1% served as controls. The virus titers of the mixtures were determined by inoculation of 10-fold dilutions into cell culture as previously described. The difference in the titer ($\log_{10}$) of the alcohol- or lipid-virus mixtures and the titer ($\log_{10}$) of the control mixture, i.e., the reduction in viral infectivity, was used as a measure of virucidal activity. Virucidal activities at low pH were measured in the same way except that the compounds were diluted in MM adjusted to pH 4.2 by addition of citrate lactate buffer (0.06 M trisodium citrate with lactic acid added to pH 4.2).

Milk products and fruit juice

Human milk samples were collected under sterile conditions 3 months postpartum and kept frozen at −86 °C until use in experiments. Low-fat milk (2% fat) and skim milk (Mjólkursamsalan, Iceland) were obtained from a local dairy store and kept at 4 °C until needed. Infant formulas where purchased from a local supplier, i.e., Mamex, Infant Formula Plus (International Nutrition Co., Denmark) and SMA Gold, First Infant Milk (SMA nutrition™, Ireland). The recommended concentrations of infant formulas for infants at age 3 months were prepared in hot sterile water according to the manufacturer's instructions and kept frozen at −86 °C until needed. Pear juice (100% from concentrate, Gerber Products Co., USA) and apple juice (Gerber) were purchased from a local supplier and kept at 4 °C until used in experiments.

Statistical analysis

The value means, standard deviations, and significant differences ($P < 0.01$) were analyzed by the Tukey–Kramer method of multiple comparison among pairs of means to determine statistical differences among different treatments [28].

Results

Inactivation of RSV by alcohols and lipids

The columns in Fig. 1 represent the infectivity titers ($\log_{10}$CCID$_{50}$) of RSV ATCC incubated with 10 mM final concentration of alcohols, fatty acids and monoglycerides for 10 min (A, B, D) and with 10 mM fatty acids for 1 min (C). Virus mixed with MM and MM with 1% ethanol served as controls. The error bars represent the standard deviation of the mean for at least three experiments for each compound. A comparison of the activities of fatty alcohols and lipids at 10 mM for 10 min against RSV ATCC showed that only one alcohol, *n*-decyl alcohol (10:0), was fully active, i.e., inactivated the virus to an undetectable level. On the other hand, 5 fatty acids, i.e., capric (10:0), lauric (12:0), myristic (14:0), palmitoleic (16:1) and oleic (18:1) acids, and 2 monoglycerides, i.e., monocaprylin (8:0) and monocaprin (10:0), were fully active, causing 4.5$\log_{10}$ or greater reduction in virus titer compared to the control (Fig. 1A, B, D). One percent ethanol had no effect on the virus titer. To further distinguish between the activities of fatty acids, they were tested for 1 min against the virus (Fig. 1C). Three fatty acids were still fully active at 1 min, i.e., lauric, palmitoleic and oleic acids. Several other compounds showed significant ($P < 0.01$) virus inactivation against RSV ATCC. For example, 10 mM lauryl (12:0) alcohol reduced the titer about 3.0$\log_{10}$ in 10 min and 10 mM monolaurin (12:0) about 3.5$\log_{10}$ in 10 min (Fig. 1).

For comparison, 3 clinical isolates of RSV were similarly tested against 10 mM concentration of alcohols, fatty acids and monoglycerides. The columns in Fig. 2 show the mean infectivity titers of all 3 strains, each tested at least 2 times. The error bars represent the standard deviation of the mean for the 3 strains. The alcohols and monoglycerides were tested for 10 min (Fig. 2A, C) and the fatty acids for 1 min (Fig. 2B). The activity profiles are nearly identical to those for RSV ATCC (Fig. 1), although the titer of the clinical isolates is only about 4$\log_{10}$ compared to 5–5.5$\log_{10}$ for the ATCC strain. The ATCC strain was therefore considered a prototype for the virus and was used in all further experiments.

Inactivation of RSV ATCC by alcohols and lipids at lower concentrations

To establish which fatty alcohols and lipids are active against RSV in a short time or at low concentrations, the compounds that were most active at 10 mM in 10 min (Fig. 1) were further tested in serial twofold dilutions. Table 1 shows the results

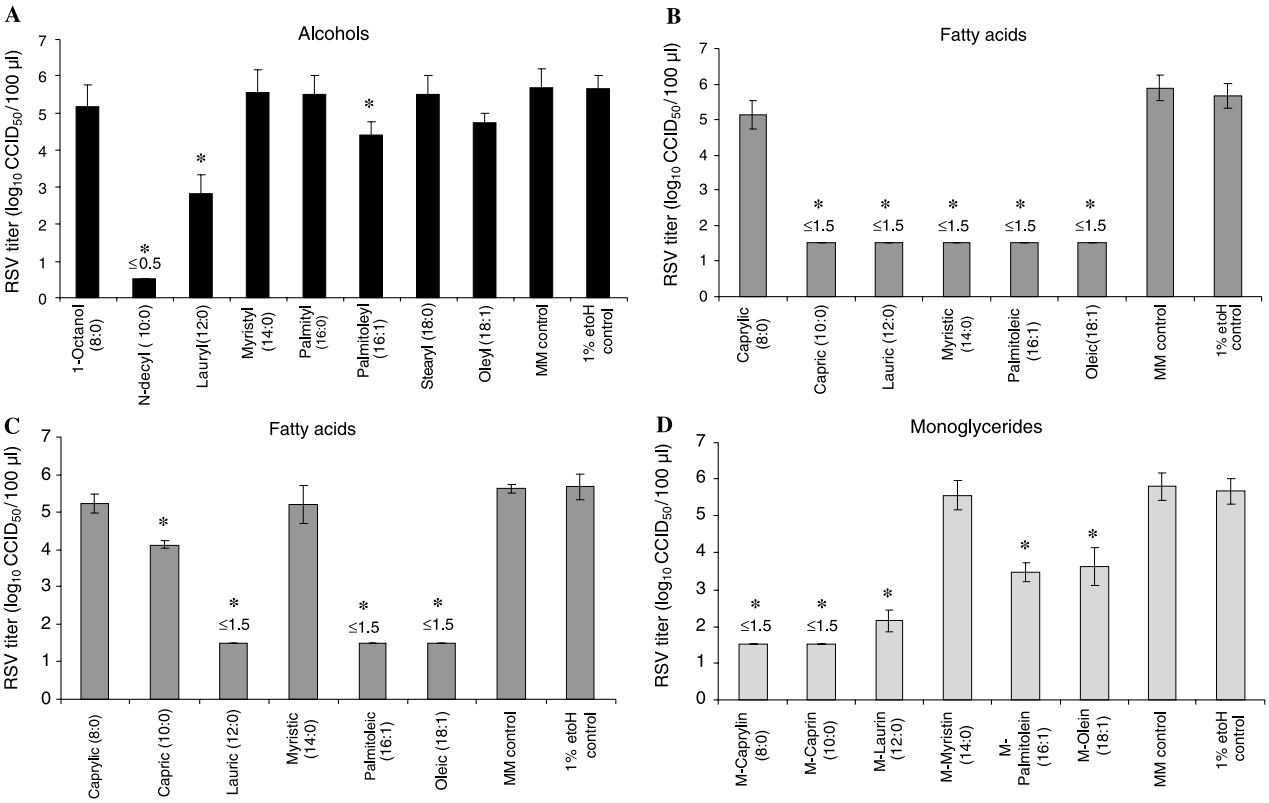

Fig. 1. Virucidal effect of fatty alcohols and lipids on RSV ATCC. Titer in $\log_{10}$ units is shown after treatment of virus with 10 mM concentration of a compound. For control the virus was mixed with medium and medium containing 1% ethanol. The error bars indicate standard deviation of the mean value for at least three experiments. At the top of a column ≤ 0.5 indicates that no virus was detectable in 100 µl of the 10^{-1} dilution, which was the lowest dilution tested and ≤ 1.5 means that no virus was detectable in 100 µl of 10^{-2} dilution, which was the lowest dilution tested due to toxic effect on the cell monolayer. Virus titer was therefore estimated as equal to or lower than $0.5\log_{10}$ or $1.5\log_{10}$. Asterisks indicate signficant reduction in virus titer compared to the controls ($P < 0.01$). (**A**) Alcohols tested for 10 min. (**B**) Fatty acids tested for 10 min. (**C**) Fatty acids tested for 1 min. (**D**) Monoglycerides tested for 10 min

after 1 min treatment. N-decyl and lauryl alcohols at 10 mM concentration had no significant effect on the virus, whereas lauric, palmitoleic and oleic acids and monocaprin were still fully active at 10 mM in 1 min, causing a $3.5\log_{10}$ or greater reduction in virus titer compared to the control, and monolaurin caused about $2.5\log_{10}$ reduction at 10 mM in 1 min. The lowest concentrations causing a significant ($P < 0.01$) reduction in virus titer were 5 mM for lauric acid, 2.5 mM for palmitoleic and oleic acids and monolaurin, and 1.25 for monocaprin, causing about $2.0\log_{10}$ reduction. Monocaprin is therefore the most active lipid against RSV in a 1-min treatment.

In a 10-min treatment (Table 2), capric acid lost its activity at 5 mM, whereas *n*-decyl alcohol was fully active against the virus at 5 mM, and lauric acid and monocaprin at 2.5 mM. Monocaprin was still highly active at 1.25 mM. Lauryl alcohol was significantly active at 0.625 mM, and monolaurin at 0.31 mM, but the activities were low. It is therefore concluded that monocaprin is the most active compound in 10 min, causing a $3.5–4\log_{10}$ reduction in virus titer at 1.25 mM concentration (Table 2).

Inactivation of RSV by fatty alcohols and lipids at different pH levels

Since the carboxyl group of fatty acids is presumably affected by acidic pH, capric and lauric acids were tested at lower concentrations against RSV

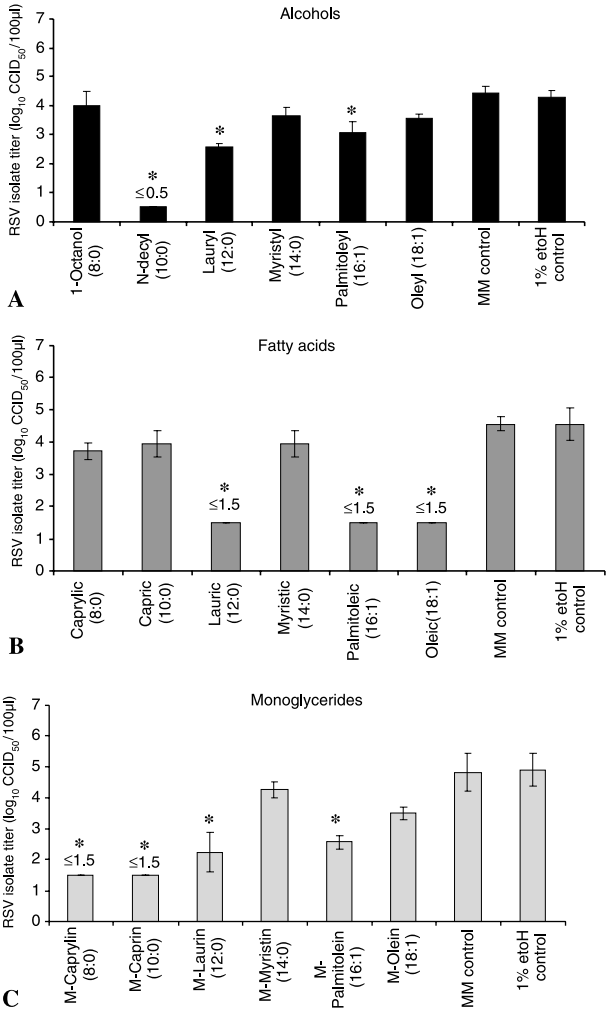

Fig. 2. Virucidal effects against three recent clinical isolates of RSV. Titer in $\log_{10}$ units is shown after treatment of virus with (**A**). 10 mM alcohols for 10 min (**B**). 10 mM fatty acids for 1 min and (**C**). 10 mM monoglycerides for 10 min. For control the virus was mixed with medium and with medium containing 1% ethanol. ≤ 0.5 indicates that no virus was detected in 100 µl of the 10^{-1} dilution which was the lowest dilution tested. ≤ 1.5 indicates that no virus was detected in 100 µl of the 10^{-2} dilution which was the lowest dilution tested due to toxic effect on the cell monolayers. The *error bars* indicate the standard deviation of the mean for all three isolates. *Asterisks* indicate significant reduction in virus titer compared to the controls ($P < 0.01$)

ATCC for 1 and 10 min at pH 7 and 4.2, respectively. The corresponding alcohols and monoglycerides were tested in the same way. The results are shown in Table 3. N-decyl and lauryl alcohols and capric acid in 10 mM concentration showed minor virucidal effects in 1 min at pH 7, but by changing to pH 4.2 these compounds became much more active. The activities of lauric acid and the monoglycerides were less increased by the acidic pH in 1 min. On the other hand, after a 10-min treatment, all of the compounds became much more active against RSV at pH 4.2 as compared with the same concentration at pH 7 (Table 3). As a matter of fact, they all changed from no or very low activities at pH 7 to being fully active at pH 4.2. Thus, at pH 4.2, monocaprin and lauric acid were fully active at 0.62 mM concentration, and lauryl alcohol and monolaurin at 0.31 mM. At pH 4.2 there was also a slight but significant inactivation of the virus control after a 10-min incubation.

Inactivation of HPIV2 by fatty alcohols and lipids at various concentrations and at different pH levels

For comparison with RSV, the 6 most active compounds were tested in a similar way against HPIV2. The results for 10-min treatment are shown in Table 4. N-decyl alcohol at 2.5 mM concentration and lauryl alcohol at 0.625 mM were still significantly ($P < 0.01$) active against this virus, which is in accordance with their effects against RSV. There were, however, some differences in the activities of the alcohols against the two viruses, particulary at 5 mM and 2.5 mM concentrations. The activities of the fatty acids and monoglycerides against the viruses were almost identical (Tables 2 and 4).

The compounds were tested in a similar way against HPIV2 for 10 min at pH 4.2 at concentrations which showed no activities at pH 7. The results are shown in Table 5. N-decyl alcohol and capric and lauric acid all became fully active against the virus at pH 4.2, similar to RSV. Lauryl alcohol showed much less increase in activity, and monocaprin and monolaurin, in contrast to RSV, showed no increase in activity at pH 4.2 compared to pH 7 (Tables 3 and 5). These compounds were also tested at higher concentrations, i.e., 0.62 mM and 1.25 mM, at pH 4.2, with no increase in activity (data not shown).

Table 1. Virucidal effects against RSV ATCC after treatment with compound for 1 min

Compound	10	5	2.5	1.25	0.625
Virus titer $\log_{10}(CCID_{50}/100\,\mu l)$[a] after treatment with different concentrations of compounds (mM)[b]					
N-decyl alcohol (10:0)[c]	4.17 ± 0.29	ND[d]	ND	ND	ND
Lauryl alcohol (12:0)	4.37 ± 0.12	ND	ND	ND	ND
Lauric acid (12:0)	≤ 1.5[e] ± NA[f]	$1.57^* \pm 0.12$	3.90 ± 0.17	ND	ND
Palmitoleic acid (16:1)	$\leq 1.5 \pm$ NA	1.67 ± 0.29	$1.83^* \pm 0.58$	3.80 ± 0.17	ND
Oleic acid (18:1)	$\leq 1.5 \pm$ NA	1.73 ± 0.25	$3.10^* \pm 0.17$	4.57 ± 0.12	ND
Monocaprin (10:0)	$\leq 1.5 \pm$ NA	$\leq 1.5 \pm$ NA	1.67 ± 0.29	$2.17^* \pm 0.58$	4.33 ± 0.76
Monolaurin (12:0)	2.57 ± 0.12	2.73 ± 0.25	$3.47^* \pm 0.68$	4.00 ± 0.87	4.40 ± 0.79
Control	5.15 ± 0.17				

[a] Mean for 3 experiments ± standard deviation.
[b] Final concentration.
[c] Number of carbon atoms:number of double bonds.
[d] *ND* Not done.
[e] ≤ 1.5 indicates that no virus was detected in 100 µl of the 10^{-2} dilution, which was the lowest dilution tested due to toxic effects on Vero cells.
[f] *NA* Not available.
* The lowest concentration of compound causing a significant reduction in virus titer compared to the control ($P < 0.01$).

Table 2. Virucidal effects against RSV ATCC after treatment with compound for 10 min

Compound	10	5	2.5	1.25	0.625	0.312
Virus titer $\log_{10}(CCID_{50}/100\,\mu l)$[a] after treatment with different concentrations of compounds (mM)[b]						
N-decyl alcohol (10:0)[c]	≤ 0.5[d] ± NA[e]	$\leq 0.5 \pm$ NA	$3.40^* \pm 0.36$	5.07 ± 0.01	ND[f]	ND
Lauryl alcohol (12:0)	2.13 ± 0.60	2.40 ± 0.17	2.63 ± 0.12	2.57 ± 0.12	$3.30^* \pm 0.53$	4.07 ± 0.12
Capric acid (10:0)	$\leq 1.5^{*,g} \pm$ NA	4.17 ± 0.29	4.47 ± 0.68	5.00 ± 0.30	ND	ND
Lauric acid (12:0)	$\leq 1.5 \pm$ NA	$\leq 1.5 \pm$ NA	$\leq 1.5 \pm$ NA	$3.40^* \pm 0.53$	4.63 ± 0.60	ND
Monocaprin (10:0)	$\leq 1.5 \pm$ NA	$\leq 1.5 \pm$ NA	$\leq 0.5 \pm$ NA	$0.83^* \pm 0.58$	4.07 ± 0.40	ND
Monolaurin (12:0)	2.07 ± 0.40	2.57 ± 0.12	2.67 ± 0.29	2.80 ± 0.53	3.03 ± 0.29	$3.23^* \pm 0.40$
Control	5.13 ± 0.12					

[a] Mean for 3 experiments ± standard deviation.
[b] Final concentration.
[c] Number of carbon atoms:number of double bonds.
[d] ≤ 0.5 indicates that no virus was detected in 100 µl of the 10^{-1} dilution, which was the lowest dilution tested.
[e] *NA* Not available.
[f] *ND* Not done.
[g] ≤ 1.5 indicates that no virus was detected in 100 µl of the 10^{-2} dilution, which was the lowest dilution tested due to toxic effects on Vero cells.
* The lowest concentration of compound causing a significant reduction in virus titer compared to the control ($P < 0.01$).

Inactivation of influenza A virus by monocaprin

Monocaprin, which has previously been shown to be one of the most active lipids, was tested in decreasing mM concentrations against the clinical isolate of influenza A virus at pH 7. The results are depicted in Table 6. Ten mM monocaprin was fully active in 1 min, causing 3.0 $\log_{10}$ or greater reduction in virus titer compared to the control. At 1.25 mM concentration, monocaprin still showed significant ($P < 0.01$) virus inactivation against the virus, with about 2.0 $\log_{10}$ reduction in only 1 min. Monocaprin was fully active after 10 min at 10, 5 and 2.5 mM concentrations and inactivated the virus to an undetectable level. It also caused signifi-

Table 3. Virucidal activities of the most active compounds at two different pH levels, tested against RSV ATCC for 1 and 10 min. Each compound was tested at a concentration at which it was not fully active at pH 7

Compound	1 min			10 min		
	mM[b]	pH 7	pH 4.2	mM	pH 7	pH 4.2
Virus titer $\log_{10}(CID_{50}/100\,\mu l)$[a]						
N-Decyl (10:0)[c] alcohol	10	4.17 ± 0.29	$\leq 1.5^{d,**} \pm NA^{e}$	2.5	$3.40^{*} \pm 0.36$	$\leq 0.5^{f,**} \pm NA$
Capric (10:0) acid	10	3.77 ± 0.50	$\leq 1.5^{**} \pm NA$	2.5	4.47 ± 0.68	$\leq 0.5^{**} \pm NA$
Monocaprin (10:0)	1.25	$2.17^{*} \pm 0.58$	1.75 ± 0.35	0.62	$4.07^{*} \pm 0.12$	$\leq 0.5^{**} \pm 0.35$
Lauryl (12:0) alcohol	10	4.37 ± 0.12	$1.57^{**} \pm 0.12$	0.31	$4.07^{*} \pm 0.12$	$\leq 0.5^{**} \pm 0.12$
Lauric (12:0) acid	2.5	3.90 ± 0.17	$2.50^{**} \pm 0.20$	0.62	4.63 ± 0.60	$\leq 0.5^{**} \pm 0.20$
Monolaurin (12:0)	1.25	4.00 ± 0.87	$2.53^{**} \pm 0.68$	0.31	$3.23^{*} \pm 0.40$	$\leq 0.5^{**} \pm 0.68$
Control		5.15 ± 0.17	5.00 ± 0.17		5.13 ± 0.12	$4.07^{*} \pm 0.12$

[a] Mean for 3 experiments ± standard deviation.
[b] Final concentration.
[c] Number of carbon atoms: number of double bonds.
[d] ≤ 1.5 indicates that no virus was detectable in 100 µl of 10^{-2} dilution, which was the lowest dilution tested due to toxic effects on Vero cells.
[e] *NA* Not available.
[f] ≤ 0.5 indicates that no virus was detected in 100 µl of the 10^{-1} dilution, which was the lowest dilution tested.
* Significant reduction in virus titer compared to the control at pH 7 ($P < 0.01$).
** Significant reduction in virus titer at pH 4.2 compared to pH 7 ($P < 0.01$).

Table 4. Virucidal activities of the most active compounds tested against HPIV2 for 10 min

Compound	10	5	2.5	1.25	0.625	0.312
Virus titer $\log_{10}(CCID_{50}/100\,\mu l)$[a] after treatment with different concentrations of compounds (mM)[b]						
N-Decyl alcohol (10:0)[c]	0.73 ± 0.25	2.73 ± 0.25	$3.97^{*} \pm 0.25$	4.90 ± 0.17	ND[d]	ND
Lauryl alcohol (12:0)	2.23 ± 0.25	$\leq 1.5^{e} \pm NA^{f}$	$\leq 1.5 \pm NA$	2.47 ± 0.64	$3.47^{*} \pm 0.25$	4.47 ± 0.25
Capric acid (10:0)	$\leq 1.5 \pm NA$	$3.47^{*} \pm 0.40$	4.87 ± 0.29	ND	ND	ND
Lauric acid (12:0)	$\leq 1.5 \pm NA$	$\leq 1.5 \pm NA$	$1.97^{*} \pm 0.40$	5.07 ± 0.12	5.07 ± 0.12	ND
Monocaprin (10:0)	$\leq 1.5 \pm NA$	$\leq 1.5 \pm NA$	$\leq 0.5^{g} \pm NA$	$2.17^{*} \pm 0.42$	4.53 ± 0.29	ND
Monolaurin (12:0)	2.27 ± 0.25	2.63 ± 0.12	3.00 ± 0.00	3.13 ± 0.12	$3.33^{*} \pm 0.29$	4.30 ± 0.17
Control	5.13 ± 0.12					

[a] Mean for 3 experiments ± standard deviation.
[b] Final concentration.
[c] Number of carbon atoms:number of double bonds.
[d] *ND* Not done.
[e] ≤ 1.5 indicates that no virus was detected in 100 µl of the 10^{-2} dilution, which was the lowest dilution tested due to toxic effects on Vero cells.
[f] *NA* Not available.
[g] ≤ 0.5 indicates that no virus was detected in 100 µl of the 10^{-1} dilution, which was the lowest dilution tested.
* The lowest concentration of compound causing a significant reduction in virus titer compared to the control ($P < 0.01$).

cant virus reduction at 0.62 mM concentration after a 10-min incubation. These results are in good agreement with the virucidal activities seen for decreasing mM concentrations of monocaprin against RSV and HPIV2 (Tables 1, 2, 4 and 6).

Inactivation of RSV by lipids mixed with milk products and fruit juice

Four lipid compounds that were previously shown to have high virucidal activities in MM against RSV were tested in human milk, low-fat (2%) milk,

Table 5. Virucidal activities of the most active compounds at two different pH levels, tested against HPIV2 for 10 min. Each compound was tested at a concentration at which it was not fully active at pH 7

Compound	mM[b]	pH 7	pH 4.2
Virus titer ($\log_{10}$ $CCID_{50}/100\,\mu l$)[a]			
N-Decyl (10:0)[c] alcohol	2.5	$3.97^{*} \pm 0.25$	$\leq 0.5^{d,**} \pm NA^{e}$
Capric (10:0) acid	2.5	4.87 ± 0.29	$\leq 0.5^{**} \pm NA$
Monocaprin (10:0)	0.62	4.53 ± 0.29	4.30 ± 0.36
Lauryl (12:0) alcohol	0.31	4.47 ± 0.25	$2.47^{**} \pm 0.12$
Lauric (12:0) acid	0.62	5.07 ± 0.12	$\leq 0.5^{**} \pm NA$
Monolaurin (12:0)	0.31	4.30 ± 0.17	3.83 ± 0.29
Control		5.07 ± 0.12	4.90 ± 0.17

[a] Mean for 3 experiments ± standard deviation.
[b] Final concentration.
[c] Number of carbon atoms: number of double bonds.
[d] ≤ 0.5 indicates that no virus was detectable in 100 µl of 10^{-1} dilution, which was the lowest dilution tested.
[e] *NA* Not available.
* Significant reduction in virus titer compared to the control at pH 7 ($P < 0.01$).
** Significant reduction in virus titer at pH 4.2 compared to pH 7 ($P < 0.01$).

Table 6. Virucidal activities of monocaprin at two different incubation times, tested against influenza A virus. Monocaprin was tested at 2-fold decreasing concentrations at pH 7

Concentration of monocaprin, mM[b]	1 min	10 min
Virus titer ($\log_{10}$ $CCID_{50}/100\,\mu l$)[a]		
10	$\leq 1.5^{c} \pm NA^{d}$	$\leq 1.5 \pm NA$
5	1.57 ± 0.12	$\leq 0.5^{e} \pm NA$
2.5	1.90 ± 0.17	$\leq 0.5 \pm NA$
1.25	$2.27^{*} \pm 0.25$	0.73 ± 0.25
0.62	3.90 ± 0.17	$3.51^{*} \pm 0.51$
Control (0)	4.50 ± 0.20	4.50 ± 0.20

[a] Mean for 3 experiments ± standard deviation.
[b] Final concentration.
[c] ≤ 1.5 indicates that no virus was detected in 100 µl of the 10^{-2} dilution, which was the lowest dilution tested due to cytotoxic effect.
[d] *NA* Not available.
[e] ≤ 0.5 indicates that no virus was detectable in 100 µl of 10^{-1} dilution, which was the lowest dilution tested.
* The lowest concentration of compound causing a significant reduction in virus titer compared to the control ($P < 0.01$).

Table 7. Virucidal effects against RSV by fatty compounds mixed with milk products and fruit juice for 10 min at 37 °C

Product	Fatty compound			
	Lauric acid (12:0)[b]	N-Decyl alc. (10:0)	Monocaprin (10:0)	Monolaurin (12:0)
Virus titer $\log_{10}$ ($CCID_{50}/100\,\mu l$)[a] after treatment with 10 mM concentration of compounds				
SMA	3.13 ± 0.40	3.93 ± 0.40	$\leq 1.50^{c*} \pm NA^{d}$	$1.63^{*} \pm 0.12$
SMA control	3.67 ± 0.29	4.13 ± 0.40	4.73 ± 0.25	4.23 ± 0.25
MAMEX	3.40 ± 0.17	4.07 ± 0.40	$1.67^{*} \pm 0.29$	$2.23^{*} \pm 0.40$
MAMEX control	3.90 ± 0.36	4.40 ± 0.17	4.33 ± 0.35	4.33 ± 0.29
Milk (2% fat)	3.56 ± 0.40	3.70 ± 0.00	$1.63^{*} \pm 0.00$	$1.63^{*} \pm 0.12$
Milk (2% fat) control	3.73 ± 0.25	4.16 ± 0.29	4.57 ± 0.12	4.13 ± 0.12
Skim milk	3.23 ± 0.25	$2.43^{*} \pm 0.12$	$\leq 1.50^{*} \pm NA$	$1.57^{*} \pm 0.12$
Skim milk control	3.46 ± 0.25	4.33 ± 0.29	4.17 ± 0.42	4.40 ± 0.66
Human milk	$1.63^{*} \pm 0.12$	$2.46^{*} \pm 0.25$	$\leq 1.50^{*} \pm NA$	$\leq 1.50^{*} \pm NA$
Human milk control	3.70 ± 0.00	3.80 ± 0.36	4.07 ± 0.40	3.80 ± 0.29
Pear juice	$1.57^{*} \pm 0.12$	$1.57^{*} \pm 0.12$	$\leq 1.50^{*} \pm NA$	$\leq 1.50^{*} \pm NA$
Pear juice control	3.30 ± 0.17	$3.40^{**} \pm 0.17$	$3.40^{**} \pm 0.17$	3.87 ± 0.29
MM	$\leq 1.50^{*} \pm NA$	$\leq 1.50^{*} \pm NA$	$\leq 1.50^{*} \pm NA$	$1.57^{*} \pm 0.12$
MM control	4.13 ± 0.12	4.47 ± 0.25	4.57 ± 0.12	4.57 ± 0.12

[a] Mean for 3 experiments ± standard deviation.
[b] Number of carbon atoms:number of double bonds.
[c] ≤ 1.5 indicates that no virus was detected in 100 µl of the 10^{-2} dilution, which was the lowest dilution tested due to toxic effects on Vero cells.
[d] *NA* Not available.
* Significant reduction in virus titer of product-fatty compound mixtures compared to product alone ($P < 0.01$).
** Significant reduction in virus titer of the product alone compared to virus control (MM control) ($P < 0.01$).

Table 8. Virucidal effects against RSV by monoglycerides at 2.5 and 1.25 mM concentrations mixed with milk products and pear juice for 10 min at 37 °C

Products	Fatty compounds		
	Monocaprin (10:0)[b] 2.0 mM[c]	Monocaprin (10:0) 1.25 mM	Monolaurin (12:0) 1.25 mM
Virus titer $\log_{10}$ ($CCID_{50}/100\,\mu l$)[a] after treatment with mixtures			
SMA	3.40 ± 0.17	ND[d]	ND
SMA control	4.30 ± 0.53	ND	ND
Skim milk	$2.57^{*} \pm 0.12$	3.63 ± 0.40	2.90 ± 0.17
Skim milk control	4.17 ± 0.29	3.67 ± 0.29	4.13 ± 0.12
Pear juice	$\leq 1.50^{e,*} \pm$ NA[f]	$\leq 0.50^{*} \pm$ NA	$\leq 0.50^{g,*} \pm$ NA
Pear juice control	3.83 ± 0.29	3.33 ± 0.29	3.13 ± 0.12
MM	$\leq 1.50^{*} \pm$ NA	$\leq 1.50^{*} \pm$ NA	$1.33^{*} \pm 0.29$
MM control	3.83 ± 0.29	4.07 ± 0.12	4.73 ± 0.46

[a] Mean for 3 experiments ± standard deviation.
[b] Number of carbon atoms:number of double bonds.
[c] Final concentration.
[d] *ND* not done.
[e] ≤1.5 indicates that no virus was detectable in 100 µl of 10^{-2} dilution, which was the lowest dilution tested due to toxic effects on Vero cells.
[f] *NA* Not available.
[g] ≤0.5 indicates that no virus was detected in 100 µl of the 10^{-1} dilution, which was the lowest dilution tested.
* Significant reduction in virus titer of product-fatty compound mixtures compared to product alone ($P < 0.01$).

skim milk, two infant formulas and pear juice. The results for lauric acid, *n*-decyl alcohol, monocaprin and monolaurin tested in 10 mM concentration for 10 min are shown in Table 7. Monocaprin and monolaurin significantly reduced the virus titer in mixtures with all of the products, compared to the products alone. For example, monocaprin reduced the virus titer about $3.0\log_{10}$ in SMA and low-fat milk. N-decyl alcohol showed less activity than the monoglycerides and was significantly active only in skim milk, human milk and pear juice. Lauric acid caused significant reduction in viral titer only in human milk and pear juice. Monocaprin and monolaurin were further tested at lower concentrations mixed with SMA, skim milk and pear juice (Table 8). It is shown that both monoglycerides were still significantly active at 1.25 mM concentration in pear juice, with about $2.5\log_{10}$ reduction in RSV titer. The milk products alone did not have a significant virucidal activity against RSV compared to the MM control. Pure pear juice (pH 3.5), on the other hand, had a significant although low virucidal activity (Table 7). Neutralized pear juice (pH 7) mixed with 1.25 mM monocaprin showed the same virucidal activity as the natural pear juice (pH 3.5). Similar results were seen for Gerber's apple juice mixed with 1.25 mM monocaprin, either pure natural (pH 3.7) or neutralized to pH 7 (data not shown).

Discussion

The comparison of virucidal activities of fatty alcohols and lipids against RSV reported in this study showed that 8 of the compounds tested at 10 mM concentration for 10 min reduced the infectivity titer up to $4.5\log_{10}$ or greater. Good agreement was found between the activity profiles for RSV ATCC and recent clinical isolates of RSV (Figs. 1 and 2). Lauric, palmitoleic and oleic acids and monocaprin all acted rapidly at 10 mM concentration, causing a significant titer reduction in only 1 min against all RSV strains (Fig. 1C and 2B, Table 1). The most active compound tested was monocaprin, which was still highly active after dilution to 1.25 mM. Although both lauryl alcohol and monolaurin could be diluted to lower concentrations without losing all activity, they were not fully active even at the highest concentration tested, i.e., 10 mM (Table 2).

A generally good agreement was seen in the virucidal activities of the 10- and 12-carbon compounds against HPIV2 and RSV (Tables 2 and 4) and also for monocaprin tested against influenza A virus in decreasing mM concentrations (Table 6 compared to Tables 1, 2 and 4). By changing the pH from 7 to 4.2, the activities of most of the compounds were greatly increased both at lower concentrations and shorter incubation time, which is in agreement with previous studies on HSV [12]. Thus, lauric acid at 0.62 mM concentration is fully active against both RSV and HPIV2 at pH 4.2 after 10 min exposure. However, there is a distinct difference in the effect of low pH on the activity of monocaprin and monolaurin against the two viruses, since, in contrast to RSV, there was no increase in the activity of these compounds against HPIV2 at pH 4.2 (Table 5).

Exactly how lipids inactivate viruses in unknown, but it has been shown that fatty acids cause disintegration of enveloped viruses [22, 23, 33]. Medium- and long-chain alcohols may act in a similiar way, i.e., penetrate the virus envelope by hydrophobic effect and inactivate the virus by causing increased permeability to small molecules. In a recent study, it was hypothesized that a difference in the hydrophile-lipophile balance (HLB) of fatty compounds could explain their different activities against viruses, but this was not confirmed since, in general, no correlation could be found between virucidal activities and HLB numbers, for example at low pH [12]. It was suggested that the increased virucidal activity against enveloped viruses at low pH may be due to ionic changes in the glycoproteins on the surface of the viral envelope, somehow giving the alcohols, fatty acids and monoglycerides better access to the lipid bilayer of the envelope. This hypothesis may be supported by the difference in the effect of low pH on the activity of monocaprin and monolaurin against RSV and HPIV2. The well-established difference in the envelope proteins of these viruses may explain the difference in their sensitivity to monoglycerides at low pH (Tables 3 and 5).

As mentioned earlier, there is a need for new prophylactic or therapeutic compounds for general use against RSV and other viruses causing respiratory infection since the drugs presently used are only recommended for high-risk children or severely ill infants, due to efficacy and safety concerns. The significant virucidal activities of fatty alcohols and lipids on RSV and HPIV2, demonstrated in this *in vitro* study, raise the question of the feasibility of using the most active compounds as ingredients in pharmaceutical dosage forms against respiratory infections caused by these viruses, and possibly other paramyxo- and myxoviruses. Several studies have indicated that lipids, particularly free fatty acids (FFAs), may serve in the natural defense of the skin and mucosal membranes against pathogens. Thus, it has been reported that FFAs play a role in the antibacterial activities of human skin [1, 18, 21], where oleic acid seemed to play the largest role. Antimicrobial lipids have been found in bronchoalveolar lavage of many animals, including humans, in lung surfactants, vaginal secretions and breast milk [8–11, 15]. Investigations indicate that they might be active in extracellular clearance in lung surfactants, and FFAs have been found in rat broncoalveolar lavage in sufficient amount to show antibacterial activities *in vitro* [9]. The idea of applying FFAs, or other lipids which kill RSV and parainfluenza virus *in vitro*, to respiratory mucosas may therefore not seem farfetched, since this might enhance a natural defense by lipids already present in the mucosa. Notably, in our study, palmitoleic and oleic acids were found to be highly active against RSV, even at low concentrations. However, because of the instability of these long-chain unsaturated fatty acids, other microbicidal compounds such as lauryl alcohol, lauric acid, monolaurin and, particularly, monocaprin may be more feasible as active ingredients in topical formulations against RSV, parainfluenza and influenza virus infections, such as nasal drops and sprays, mouth and throat rinses, and even bronchial sprays.

Hydrogels have been formulated which contain monocaprin at the highly virucidal concentration of 20 mM (0.5%) [17]. They do not cause irritation in the vaginal mucosa of mice and rabbits [19, 31], in the skin of hairless mice [18], or in the vaginal mucosa of healthy human volunteers [2] even though they are toxic in cell culture. Also, preliminary results show that monocaprin in virucidal con-

centration causes no irritations in the lungs or other physical side effects in intranasally treated mice (unpublished data). *In vivo* studies of pharmaceutical dosage forms containing monocaprin and lauric acid as active virucidal ingredients are now in preparation, using an experimental rat model intranasally infected with RSV.

It is also noteworthy that monoglycerides of fatty acids are classified as GRAS (generally recognized as safe) by the U.S. Food and Drug Administration [30] and are approved as food additives by the EU (E471). In view of the need for new pharmaceutical approches to treat RSV and other respiratory infections, it may, as outlined above, be feasible to use various lipids as active virucidal ingredients either alone or as a mixture of 2 or more compounds, and the fact that the virucidal activity can be increased in an acidic environment may be helpful in the design of such pharmaceutical formulations. In this context it is of interest that, in the present study, 4 of the most active fatty compounds were found to preserve their activities against RSV to varying degrees after mixture with milk products and fruit juice (Table 7). The experiments revealed that all 4 compounds are fully active in pear juice, and monolaurin and monocaprin are also active in various milk products. The monoglycerides were fully active in pear juice as well as in apple juice at a concentration as low as 1.25 mM, or about 0.03%. Both monocaprin and monolaurin could therefore possibly be used as food additives to protect the oral, nasal and respiratory mucosas against viral infection. It is notable that the virucidal activities of the compounds are better maintained in the juices than in the milk products. Most likely, the fatty compounds, particularly fatty acids and alcohols, bind to proteins and/or lipids in the milk, which partly block their activity [15]. This study confirms and extends previous work which suggested the possibility of protecting infants against infection by adding antimicrobially active food substances, such as antimicrobial lipids, to their diet [16].

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